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Epstein-Barr Virus Infection and Altered Control of Apoptotic Pathways in Posttransplant Lymphoproliferative Disorders

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Key Words
Posttransplant lymphoproliferative disorders · Transplantation · Epstein-Barr virus · Apoptosis

Abstract
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Introduction

Posttransplant lymphoproliferative disorders (PTLD) represent a spectrum of lymphoid diseases complicating the clinical course of transplant recipients [1–3]. Most PTLD are of B cell lineage and frequently arise from extranodal sites and at different time points after transplantation [3, 4]. The risk factors include the solid organ recipient's age, the type of organ transplanted, the host Epstein-Barr virus (EBV) infection status before transplantation, and the immunosuppressive therapy [4, 5]. PTLD are clinically, morphologically, and biologically heterogeneous diseases, including early EBV-linked, mostly polyclonal lesions and true, monoclonal EBV-positive or negative lymphomas [5]; these different lesions are classified according to the current 2008 World Health Organization Classification of Hematopoietic and Lymphoid Tumors into early lesions including infectious mononucleosis-like lesions (IM) and plasmacytic hyperplasia, polymorphic PTLD, and monomorphic PTLD reaching the criteria of lymphoma [3, 5–8]. In earlier studies, 44–90% of PTLD were reported to be EBV associated, and analysis of EBV latent gene expression identified latency type III infection in almost all EBV-infected PTLD [9, 10]. EBV is an oncogenic gamma herpesvirus that asymptomatically infects more than 90% of the general population. In vitro studies have evidenced the ability of EBV to infect and transform primary B cells into lymphoblastoid cell lines (LCLs) inducing a pattern of viral gene expression known as latency type III [11]. It has been demonstrated that only 6 of the viral latency proteins (EBNA1, –2, 3A, –3C, LP, and LMP1) are essential for efficient transformation of B cells into LCLs [11, 12]. The first viral gene to be transcribed after infection is EBNA2, which is a potent trans-activator of LMP1 and LMP2A, and numerous cellular genes, including cMYC [11, 13]. LMP1 is functionally similar to CD40, acts as a constitutively activated receptor, and can activate NF-kb signaling and downstream genes, such as the antiapoptotic Blf-1 gene and Bcl-xL [13]. Moreover, Bcl-2 (B cell lymphoma 2), an antiapoptotic protein expressed in a broad range of lymphomas, is upregulated in vitro by LMP-1. Bcl-2 family members are critical regulators of programmed cell death [14]. Deregulation of Bcl-2 family proteins has been implicated in the development of many malignancies, and it has been shown to be related to tumor progression, poor prognosis, and clinical resistance to anticancer therapy [15]. Bcl-2 proteins can be divided into three groups: antiapoptotic members (including Bcl-2, Bcl-xL, and Mcl-1), proapoptotic proteins (including Bax and Bak) directly involved in the release of apoptotic factors from the mitochondria, and the so-called BH3-only proteins (which share homology only within the third Bcl-2 homology domain, BH3) [16]. The BH3-only proteins may interact with and inhibit the antiapoptotic activity of Bcl-2 or Bcl-xL or directly activate the proapoptotic Bax or Bak proteins [17]. At least 9 mammalian BH3-only proteins have been identified to date (Bad, Bik, Blk, Hrk, Bid, Bim, Noxa, PUMA, and Bmf) [18]. Among these BH3-only proteins, Bim (Bcl-2 interacting mediator of cell death) plays a major role in the control of apoptosis in immune cells (including normal and tumoral lymphocytes) [19].

Bim activity may be regulated at both the transcriptional level and the posttranscriptional level [20]. Three different pathways of Bim regulation were recently reported in experimental models of EBV+ lymphoproliferations [21–23]. Clybouw et al. [21] found that EBV infection can lead to downregulation of the proapoptotic molecule Bim in Burkitt lymphoma cell lines via ERK activation. Inomata et al. [22] reported an additional mechanism of apoptotic pathway regulation; their work showed that microRNA are involved in cell survival regulation, including Bim expression, and that unbalanced miRNA synthesis is frequently found in lymphoproliferations. In 2009, Paschos et al. [23] reported that some specific EBV proteins (such as EBNA3A and 3C) could directly induce Bim suppression by promoter methylation. In spite of these advances in EBV-host interaction in vitro, few studies have been carried out on EBV-associated human lymphoma tissues. To gain insight into EBV-linked tumorigenesis, we investigated posttransplant lymphoproliferative diseases as models to understand the viral-host interaction in tumor development. We therefore analyzed the protein expression of pro- and antiapoptotic molecules by immunohistochemistry. In particular, we tested in a series of adult and pediatric PTLD the following molecules possibly targeted by viral products: Bcl-2, Bcl-xl, Mcl-1 (antiapoptotic); Bak, Bax, Bid, Bim, and PUMA (proapoptotic), and cleaved PARP (Poly-ADP RibosePolymerase), one of the main cleavage targets of caspase 3, a marker of effective apoptosis.

Materials and Methods

Patients

Lymphoproliferations from a total of 60 organ transplant recipients with a diagnosis of PTLD were studied. They were retrieved from the files of three institutions as follows: 27 cases from the Institute of Surgical Pathology, University Hospital Zürich (Zürich, Switzerland), 25 cases from Bicêtre/Paul Brousse Univer-

Table 1. Panel of monoclonal antibodies used in the study

Antigen	Manufacturer	Dilution	Pretreatment
EBV latency proteins			
LMP1	Dako	1/80	citrate 40'
EBNA2	Dako	1/100	EDTA 40'
Antiapoptotic proteins			
Bcl-2	Dako	1/100	EDTA 40'
Bcl-xl	ZYMED	1/50	EDTA 40'
Mcl-1	Santa Cruz	1/500	EDTA 30'
Proapoptotic proteins			
Bax	Dako	1/500	EDTA 40'
Bak	BD	1/250	EDTA 40'
PUMA	Cell Signaling	1/400	EDTA 30'
Bim	ABcam	1/400	EDTA 30'
Bid	Santa Cruz	1/100	EDTA 30'
Cleaved PARP	ABcam	1/100	EDTA 30'

sity Hospitals (Le Kremlin-Bicêtre and Villejuif, France), and 8 cases from Pavia University Hospital (Pavia, Italy). The study was accepted by the respective local ethical authorities. We focused exclusively on B cell PTLD.

Histopathology, Immunohistochemical Analysis, and EBV in situ Hybridization

All tumor specimens were formalin fixed and paraffin embedded. Prior to the study, hematoxylin and eosin (H&E)-stained whole sections were reviewed by four pathologists, and PTLD were classified according to WHO classification criteria [3]. Monoclonal antibodies against proapoptotic proteins were applied for immunohistochemistry using standard protocols (table 1). The EBV latency status was established by applying antibodies against LMP1 and EBNA2 in addition to EBER in situ hybridization (see below) (table 1). Pretreatment was customized according to the manufacturer's instructions (table 1) and performed in a microwave on whole sections and/or TMA. Immunodetection was performed with EnVision™ + Dual Link System-HRP (Dako, Glostrup, Denmark) employing 3,3′-diaminobenzidine/H_2O_2 as a chromogen. Each case was evaluated independently (M.R.G. and T.R.) for the percentage of positive cells by visual estimation and recorded in 10% increments for each antibody. Disagreements were resolved by joint review on a multi-head microscope. PTLD were considered positive when at least 20% of tumor cells expressed pro- or antiapoptotic proteins.

The EBV status was assessed by in situ hybridization (ISH) analysis using a fluorescein isothiocyanate (FITC)-labeled peptic nucleic acid (PNA) probe, complementary to the EBV-encoded RNAs (EBERs) (PNA ISH Detection Kit; Dako).

Statistical Analysis

Statistical association between protein expression in EBV-positive versus negative PTLD was tested using a two-sided Fisher's exact test and Bonferroni correction. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the software package SPSS® (version 12.0.1 for Windows®; ©SPSS Inc., Chicago, Ill., USA).

Table 2. Comparison of clinical features between EBV-positive and EBV-negative PTLD

Clinical features	EBV+	EBV–
Sex		
Male	29	12
Female	14	5
Age, years		
Median	40	63
Range	1–63	35–75
<18	12	0
≥18	31	17
Interval, months		
Median	22	52
Onset		
Early	19	0
Late	24	17
Transplant		
Liver	14	12
Heart	10	3
Lung	6	1
Kidney	7	1
Kidney/pancreas	2	0
Kidney/liver	1	0
Kidney/heart	0	1
Heart/lung	1	0
Bone marrow	1	0
Tumor site		
Lymph node	12	11
Lung	8	1
Small bowel	7	3
Liver	5	2
Kidney	3	0
Heart	2	0
Soft tissue	1	0
Adrenal gland	1	0
Brain	1	0
Peritoneum	1	0
Nasal cavity	1	0
Skin	1	0
Histology		
DLBCL	20	15
BL	2	0
P-PTLD	19	2
IM-like	2	0

Results

Patients

The clinical features of the patients with B cell PTLD included in this study are summarized in table 2. Twelve children or adolescents (<18 years of age) and 48 adults (19 female and 41 male patients) were recipients of different organ transplants. Most patients (54; 90%) received

Table 3. Upregulated apoptotic proteins in relation to EBV status, independent of the latency type

	Positive PTLD (number of cases/total number of analyzed cases), %		p value	p value (Bonferroni corrected)
	EBV–	EBV+		
Proapoptotic				
Bak	88 (15/17)	92 (35/38)	0.639	5.754
Bax	65 (11/17)	54 (22/41)	0.564	5.073
Bid	88 (15/17)	100 (42/42)	0.079	0.715
Bim	81 (13/16)	15 (6/40)	5.251×10^{-6}	4.726×10^{-5}
Effector				
PARP	71 (12/17)	23 (10/43)	0.001	
Puma	28 (5/17)	28 (12/43)	1	9
Antiapoptotic				
Bcl-2	53 (9/17)	70 (30/43)	0.243	2.188
Bcl-xl	24 (4/17)	5 (2/40)	0.058	0.525
Mcl-1	100 (17/17)	100 (43/43)	1	9

The number of evaluable cases varied due to loss of tissue or unstainability.

single organ transplantation, whereas 5 patients got double solid organ transplantation and 1 patient received an allogeneic bone marrow transplantation. Among patients with single organ transplantation, the organ most frequently transplanted was the liver (26; 48%), followed by the heart (13; 24%), kidney (8; 15%), and lung (7; 13%).

The median age at transplantation was 40 years (range 1–75). The latency period between transplantation and PTLD onset ranged from 1 to 168 months. According to the definition of Armitage et al. [7], 19 PTLDs were classified as early onset PTLD (≤12 months from transplantation) and the remaining 41 as late onset PTLD (>12 months).

Histology

The histopathological diagnoses according to 2008 WHO criteria are summarized in table 2. Most of B-PTLD in this series were monomorphic (37 cases, 62%) and high grade with a prevalence of classical DLBCL as well as two Burkitt lymphomas (BL). Twenty-one cases (35%) represented polymorphic PTLD (P-PTLD) consisting of a spectrum of B cells including mature B lymphocytes, lymphoplasmacytoid cells, plasma cells, and immunoblasts, and the two remaining cases (3%) were consistent with early lesions with an infectious mononucleosis-like pattern (IM), such as paracortical expansion and numerous immunoblasts in a background of T cells and plasmocytes.

Immunohistochemical Findings

EBV Status and Latency Pattern

Forty-three of 60 (72%) PTLD were EBV positive as determined by the expression of EBERs. The EBV latency type was assessed by LMP1 and EBNA2 in 24 EBV+ cases. Twenty-one EBV+ PTLD case showed a type III (LMP1+, EBNA2+) latency pattern (88%), 1 EBV+ PTLD case showed type II (LMP1+, EBNA2–) latency, and the 2 remaining EBV+ PTLD cases showed a type I (LMP1–, EBNA2–) latency pattern.

EBV Status and Disease Onset

The time period between transplantation and PTLD onset was much shorter in EBV-positive cases than in negative cases, with a median interval of 22 months (range 2–156) in the EBV-positive cases compared to a median of 52 months (range 21–168) in the EBV-negative ones.

Expression of Apoptosis-Related Molecules

The immunohistochemical findings in relationship to EBV status are summarized in table 3.

In the EBV-positive PTLD group, Bcl-2 was expressed in a total of 30 of 43 cases (70%). Only 2 of 40 lymphomas (5%) were positive for Bcl-xl and all analyzable PTLD expressed Mcl 1. Twenty-two of 41 cases (54%) expressed Bax; 35 of 38 cases (92%) expressed Bak, only 6 of 40 PTLD cases (15%) expressed Bim (fig. 1), and 12 of 43 PTLD cases (28%) expressed PUMA. All analyzable cases expressed Bid. Cleaved PARP was found to be positive in 10 of 43 cases (23%).

In the EBV-negative PTLD group, antiapoptotic proteins were expressed as follows: Bcl-2 in 9 of 17 cases (53%), Bcl-xl in 4 of 17 cases (24%), and Mcl-1 in all cases (100%). The proapototic proteins Bax, Bim, Puma, Bid, and Bak were respectively expressed in 11/17 cases (65%), 13/16 cases (81%), 5/17 cases (29%), 15/17 (88%), and 15/17 (88%); cleaved PARP was positive in 12 cases (71%).

Taken together, the expressions of the proapoptotic protein, BH3 only, Bim, and the apoptosis effector molecule cleaved PARP were significantly ($p < 0.05$ by Fisher's exact test and Bonferroni correction) downregulated in EBV-positive PTLD compared to EBV-negative ones. On the contrary, the expression of antiapoptotic proteins (Bcl2, Bcl-xL, and Mcl1) as well as the remaining proapoptotic proteins such as Bax and Bak Puma, Bad, and Bid did not show any significant differences ($p < 0.05$).

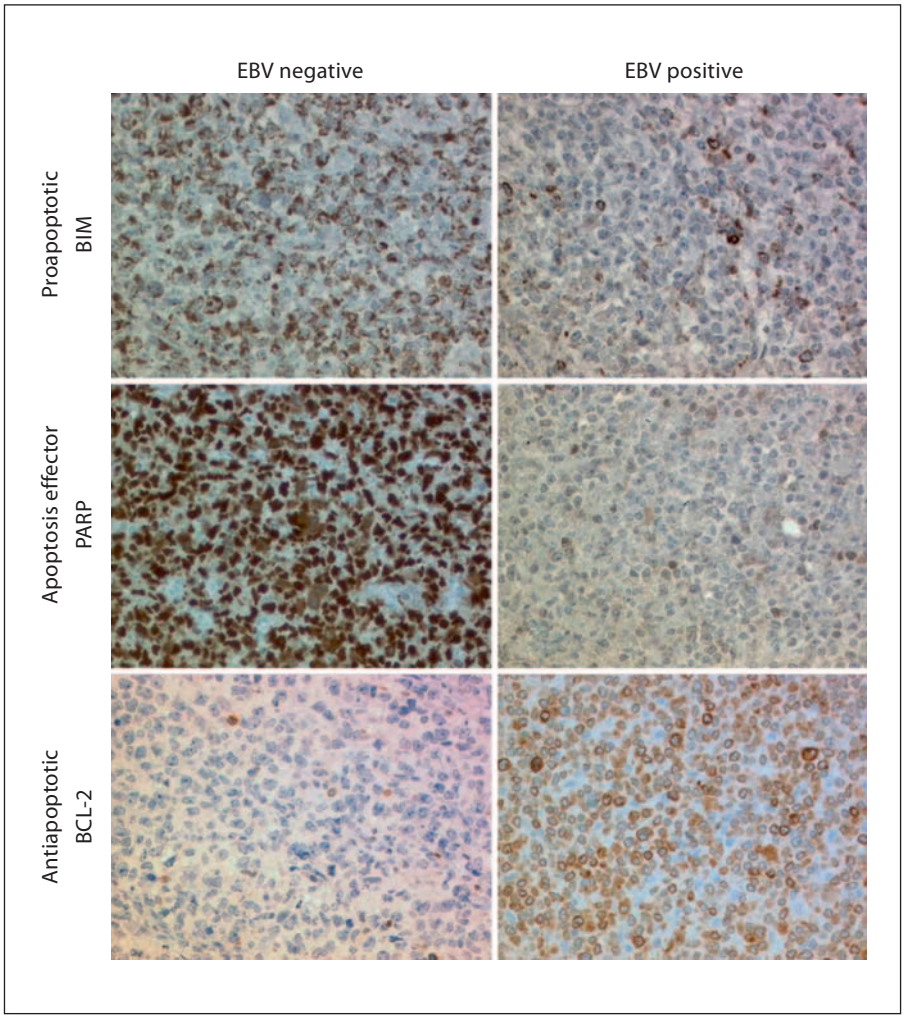

Fig. 1. Summary of the characteristic staining pattern in EBV-positive versus EBV-negative monomorphic PTLDs (DLBCL). EBV-negative example showing a high expression of the proapoptotic proteins BIM and cleaved PARP and no expression of the antiapoptotic protein Bcl-2, whereas the opposite is true for EBV-positive PTLD.

Interestingly, however, there was at least a tendency toward a higher Bcl-2 expression in EBV-positive compared to EBV-negative PTLD ($p = 0.24$). Particularly, Bcl-2 was expressed in a high percentage of EBV latency type III (87%) cases as compared to latency I and latency II types or EBV negative lymphoproliferations ($p = 0.47$). This latter observation underlines the role of EBV in Bcl-2 upregulation since most PTLD in this series are indeed of latency type III.

Discussion

In this study we analyzed a large series of sixty B cell PTLD, the majority (72%) of which were EBV associated as determined by EBER in situ hybridization. Although it is well established that EBV plays a crucial role in the development of PTLD, the mechanism of interaction with the host cells was fairly unknown in humans. We investigated 9 pro- and antiapoptotic proteins in primary lymphoma tissues. By immunohistochemistry we revealed a distinct staining pattern in the EBV-positive group with a low-expression pattern of the proapoptotic protein Bim and the apoptosis effector cleaved PARP as well as a high-expression pattern of Bcl-2, particularly in EBV latency type III cases. Hence, our results strongly suggest that EBV promotes prosurvival signals in host cells by interfering with the apoptosis pathway. Interestingly, the staining pattern is independent of PTLD histology, the time lag between disease onset and transplantation, patient age, and transplanted organs, suggesting a robust association with the viral infection. The most important role seems to be advocated to Bim, which is a critical regulator of lymphocyte survival, and

its reduced expression is associated with lymphomagenesis in mice and humans [24]. The monoclonal antibody used in our study was generated using a synthetic peptide corresponding to residues in exon 2 of human Bim. It recognizes all known splice variants of Bim, but no other Bcl-2 family members. In normal tonsil B cells, the three major Bim isoforms are strongly associated with the antiapoptotic Bcl-2 family members Mcl-1, Bcl-2, and Bcl-xL [25]. The Mcl-1/Bim complex is the most abundant among the three types of complexes [26]. Modification of the Mcl-1/Bim complex might be caused by modification of Bim and/or Mcl-1 protein levels or by a complete or partial dissociation of the Mcl-1/Bim dimer [27]. In our PTLD series, Mcl-1 was found to be equally expressed in all analyzable PTLD, independently of the EBV status. This is an additional argument in favor of prosurvival signals since Mcl-1 expression is crucial for the development and viability of hematopoietic cells and a decrease in its expression leads to cell death [28]. Moreover, it supports the assumption of a disrupted apoptotic balance specifically targeting Bim in EBV+ PTLD.

Several control mechanisms downregulating Bim expression are currently known: through ERK phosphorylation and proteasome degradation [21], through the miRNA system [22], and via the EBV proteins EBNA3A and 3C through promoter gene methylation [23]. It is worth mentioning that in our series all EBV+ PTLD showing a low-expression pattern of Bim displayed an EBV latency type III program, suggesting a role of EBNA3A and 3C in methylation of the Bim promotor.

In this study, we analyzed the expression of cleaved PARP as an effector of apoptosis control. Cleaved PARP is involved in DNA repair and maintenance of genomic integrity, regulation of protein expression through a posttranscriptional mechanism (such as inflammatory mediators), and apoptosis, through caspase cleavage [29]. Depending on the severity of the DNA damage, genotoxic stimuli can trigger different pathways; whereas in mild DNA damage cleaved PARP induces DNA repair and thus survival, more severe DNA damage provokes apoptotic cell death by the induction of caspases cleavage leading to fragmentation of cleaved PARP into two subunits (p89 and p24) [30]. In our series, a low percentage of cells expressing cleaved PARP was observed in EBV-positive PTLD, reflecting a decreased level of apoptosis in this group.

Puma, a proapoptotic protein transcriptionally upregulated by p53 and activated by p53-dependent apoptotic stimuli did not reach any significant difference between the two PTLD groups (12 of 43 EBV+ PTLD and 5 of 17 EBV– PTLD Puma-positive cases). However, a tendency toward lower expression levels in the EBV-positive group was observed. This tendency is concordant with lowered Bim expression in the presence of EBV.

Bcl-2, an antiapoptotic protein, showed a tendency toward increased expression in EBV-positive PTLD in general and of EBV latency type III in particular in our series. This is in line with the notion that Bcl-2 is upregulated in immortalized lymphoblastoid B cells of EBV latency type III and in EBV-positive PTLD via LMP1 [31, 32]. However, for restriction of tissue, EBV latency could be assessed in only half of the EBV-positive PTLD in our study. For this reason, we are unable to draw affirmative conclusions on the putative influence viral proteins and particularly LMP1 might have on Bcl-2 expression of EBV carrying tumor cells. Moreover, Bcl-2 protein was detectable in 53% of EBV-negative cases, suggesting that different mechanisms participate in its upregulation in PTLD. Nevertheless, given its antiapoptotic function and its role in conferring resistance to chemotherapeutic agents, Bcl-2 represents an interesting therapeutic target in the PTLD setting. Notably, it was shown that Bcl-2 antisense enhances the in vitro and in vivo response of EBV-associated lymphoproliferative diseases to the anti-CD 20 antibody rituximab [33], a treatment which is frequently considered in PTLD patients and has been shown to be effective in other B cell malignancies (reviewed by Masood et al. [34]). Recent studies on new generation molecules with anti-Bcl-2 reactivity report on their potentiating role of chemotherapeutic activity, as well as on anti-CD 20 antibody, making this easily applicable molecule an ideal predictive marker [35].

In summary, we demonstrate a specific expression pattern of apoptosis pathway-related molecules promoting cell survival in EBV-infected PTLD cells. Our results further suggest an important role of the proapoptotic protein Bim in the disruption of apoptosis by EBV.

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